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Quantification of valproic acid and its metabolite 2-propyl-4-pentenoic acid in human plasma using HPLC-MS/MS

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Abstract

A specific and sensitive HPLC-MS/MS method for the quantitative determination of valproic acid (VPA) and its metabolite, 2-propyl-4-pentenoic acid in human plasma has been developed, using VPA-d_{15} as the internal standard. The method was based on pre-column derivatization using 4-dimethylaminobenzylamine dihydrochloride. The derivatives were separated with a gradient elution and quantified by positive electrospray ionization with multiple reaction monitoring. The assay provides routine quantification limits of 200 ng/mL for VPA and 20 ng/mL for 4-ene VPA with within- and between-day coefficients of variation of <10%. This method has been applied to the analysis of plasma samples obtained from patients treated with this drug.

Keywords: Valproic acid; Metabolites; Derivatization; LC-MS/MS; Clinical application

1. Introduction

Valproic acid (VPA, 2-propylpentanoic acid) (Fig. 1), a branched C8 carboxylic acid, is commonly used for the treatment of seizures and as a mood stabilizing agent for variety of psychiatric disorders [1–3]. Recent reports showed that VPA has anti-tumor activity likely mediated by inhibition of histone deacetylase (HDAC) activity, which induces gene silencing via aberrant chromatin remodeling [4,5]. *In vivo* as a single agent or in combination with differentiating agents, VPA has shown clinical activity in patients with myelodysplastic syndromes (MDS) [6]. However, a direct correlation between VPA levels, pharmacodynamic endpoints (i.e., histone hyperacetylation and levels of HDAC inhibition), toxicity (i.e., sedation, confusion and hepatic dysfunction) and clinical response has not been determined [7,8]. It is therefore important to establish a sensitive and accurate analytical method in order to monitor levels of the parent drug and its metabolites and to facilitate correlation of drug levels with clinical and biological endpoints.

Due to the volatile nature of small molecules in general, direct quantification of VPA and the corresponding metabolites has proven to be challenging. Gas chromatographic-mass spectrometry (GC-MS) and high performance liquid chromatography (HPLC) methods have been established and modified for quantification of VPA in attempts to increase the sensitivity, albeit only with modest success [9–12]. Additionally, none of these studies provide validation data for quantification of VPA metabolites. We developed and validated a sensitive, and specific liquid chromatographic-tandem mass spectrometry (LC-MS/MS) method for the simultaneous quantification of VPA and a major metabolite 2-propyl-4-pentenoic acid (4-ene VPA) in human plasma (Fig. 1). This method has been applied to characterize the preliminary VPA pharmacokinetics in patients with acute myeloid leukemia (AML) treated with VPA in combination with the hypomethylating agent decitabine.

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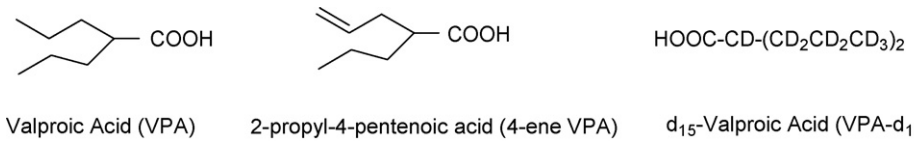


Fig. 1. Structure of valproic acid (VPA), 2-propyl-4-pentenoic acid (4-ene VPA) and VPA-d_{15} (I.S.).

2. Experimental

2.1. Chemical and reagent

Non-formulated sodium valproate, 2-chloro-1-methylpyridinium iodide and 4-dimethylaminobenzylamine dihydrochloride were purchased commercially (Sigma, St. Louis, MO, USA). 4-ene VPA was purchased from Narchem Corp (Chicago, IL, USA) and VPA-d_{15} was obtained from CDN Isotope Inc. (Pointe-clair, Quebec, Canada). Acetonitrile, methanol, dimethylchloride, triethylamine, buffer solution (pH 4) and formic acid were of analytical grade and purchased from Fisher Scientific (Pittsburgh, PA, USA). Distilled water was purified using an E-pure water purification system (Barnstead, Dubuque, IA, USA). All chemicals and reagents were used as received.

2.2. Instrumentation

A Perkin-Elmer Sciex API 300 triple quadrupole mass spectrometer (Thornhill, Ontario, Canada) coupled to a Shimadzu HPLC system (Shimadzu, Columbia, MD, USA) was used. The HPLC system was equipped with an SCL-10A system controller, an LC-10AD pump and a SIL-10A autosampler.

2.3. Chromatographic conditions

A gradient elution system was used in the determination of VPA and 4-ene VPA derivatives concentration in human plasma. The mobile phase used consisted of A, acetonitrile/water 20/80 (v/v) containing 0.1% (v/v) formic acid and B, consisted of acetonitrile/water 70/30 (v/v) containing 0.1% (v/v) formic acid. The mobile phase was delivered at a flow rate of 0.2 mL/min to a Thermo Beta Basic C8 5 μm column (50 cm × 2.1 mm) (Thermo Hypersil-Keystone, Bellefonte, PA), which was coupled to a 2 μm precolumn filter (Thermo Hypersil-Keystone). The elution was initiated with 100% A for 11 min and changed to 100% B in 1 min. The elution was kept at 100% B for 4 min before returning to 100% A in 1 min and then equilibrated at 100% of A for 3 min. The total run time was 20 min. The LC eluate was introduced into the API source at 10 μL/min after a 95:5 (LC:MS) split.

2.4. Mass spectrometry

The mass spectrometer was operated using electrospray ionization (ESI) with an ionspray voltage of +4200 V. The positive ion multiple-reaction-monitoring (MRM) mode analysis was performed using nitrogen as the collision gas. The curtain gas (nitrogen) flow and the ionspray flow were set at 0.6 and 0.9 L/min, respectively. The pressure in the collision cell was set at 0.29 Pa. The orifice voltage and ring voltage were set to +20 and +250 V, respectively. A dwell time of 300 ms and a pause time of 5 ms between scan were typically used to monitor precursor/product ion pairs of the VPA derivative (2-propyl-pentanoic acid 4-dimethylamino-benzylamide), the d_{15}-VPA derivative (I.S.), and the 4-ene VPA derivative (2-propyl-4-pentenoic acid 4-dimethylamino-benzylamide). The mass spectrometer was tuned to its optimum sensitivity and mass accuracy by infusion of a standard calibration solution of polypropylene glycol (PPG) on a daily basis. This tuning was further adjusted by injection of a fresh standard solution of VPA derivative at 5 ng/mL in the HPLC mobile phase as described above. Mass spectrometry conditions were optimized using a syringe pump infusion (5 μL/min).

2.5. Sample preparation procedure

With a serial dilution of stock solution, the working standards or working quality control solutions were prepared. Ten microlitres of working standards or working quality control solution were spiked into a 90 μL blank plasma sample to give final concentrations of 0, 20, 50, 100, 200, 500, 1000, 2000 and 5000 ng/mL. Ten microlitres of working internal standard was also spiked to the sample. The samples were acidified to pH 3 with hydrochloric acid solution (3N) and extracted with methylene chloride (1000 μL, 30 min) using a mechanical shaker for 30 min. After centrifugation at 15,900 × *g* for 1 min, the organic layers were transferred to new 1.7 mL Omniseal microtubes (Life Science Products, Inc.) and 100 μL 2-chloro-1-methylpyridinium iodide (10 mg/mL)/TEA (40 mg/mL) (1:1, v/v) was added. Following thorough mixing, 100 μL 4-dimethylaminobenzylamine dihydrochloride solution (10 mg/mL)/TEA (1:1, v/v) was added. The solution was shaken at 37 °C for 1 h before an addition of 500 μL buffer (pH 4). The mixture was mixed by vortex for 1 min and centrifuged at 15,900 × *g* for 1 min. The organic layer was separated and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 120 μL of acetonitrile/water (20:80, v/v) containing 0.1% HCOOH, and a 40 μL aliquot was introduced for LC-MS/MS analysis.

2.6. Assay validation

The linearity of the method was evaluated by an analysis of plasma standard curve samples, which were prepared by spiking the working standard and working internal standard solutions to blank human plasma (American Red Cross, Columbus OH, USA). The concentrations of VPA in the final samples were 0, 200, 500, 1000, 2000 and 5000 ng/mL and those of 4-ene VPA were 0, 20, 50, 100, 200 and 500 ng/mL. Calibration curves

were generated by plotting peak area ratios between analyte and I.S. against concentration. The within-day/between-day accuracy and precision were evaluated by analyzing six replicates of spiked plasma samples at three concentration levels for each. The quality control (QC) samples were prepared the same procedure as for standard curve samples. For VPA, QC-low (200 ng/mL), QC-medium (1000 ng/mL), and QC-high (5000 ng/mL) were analyzed. For 4-ene VPA, QC-low (20 ng/mL), QC-medium (50 and 100 ng/mL), and QC-high (200 ng/mL) were used. The accuracy of the assay was determined by comparing the nominal concentrations with the corresponding calculated concentration via linear regression.

2.7. Interference test

To evaluate if the presence of high concentrations of sodium VPA would influence the analysis of 4-ene VPA, the interference study was performed. Ten microlitres of working standard solution each of 4-ene VPA were spiked into 90 μL blank plasma sample containing 100 μg/mL VPA to give final concentrations of 0, 20, 50, 100, 200 ng/mL and 10 μL working internal standard was spiked into the sample. The samples were extracted and derivatized similarly.

2.8. Clinical pharmacokinetic study in AML patients

Plasma samples were obtained from patients with AML enrolled on OSU Protocol 0336 for treatment with decitabine and VPA in combination. This protocol was approved by the Institutional Review Board of The Ohio State University and the samples were collected following patients' informed consent. Patients received 15, 20 or 25 mg/kg/day VPA orally in three divided doses. Blood samples were collected at time points of pre-dose, 5, 10, 30, 60, 65, 75, 90, 120, 150, 180, 240, 360 and 480 min. The plasma was separated by centrifugation and kept at −80 °C until analysis.

3. Results and discussion

The primary objective of this study was to develop a sensitive method for *in vivo* quantification of VPA and its metabolite 4-ene VPA. Due to the lack of a good chromophore in VPA, an HPLC-UV assay is not expected to have adequate sensitivity for the analysis of VPA in biological samples. The

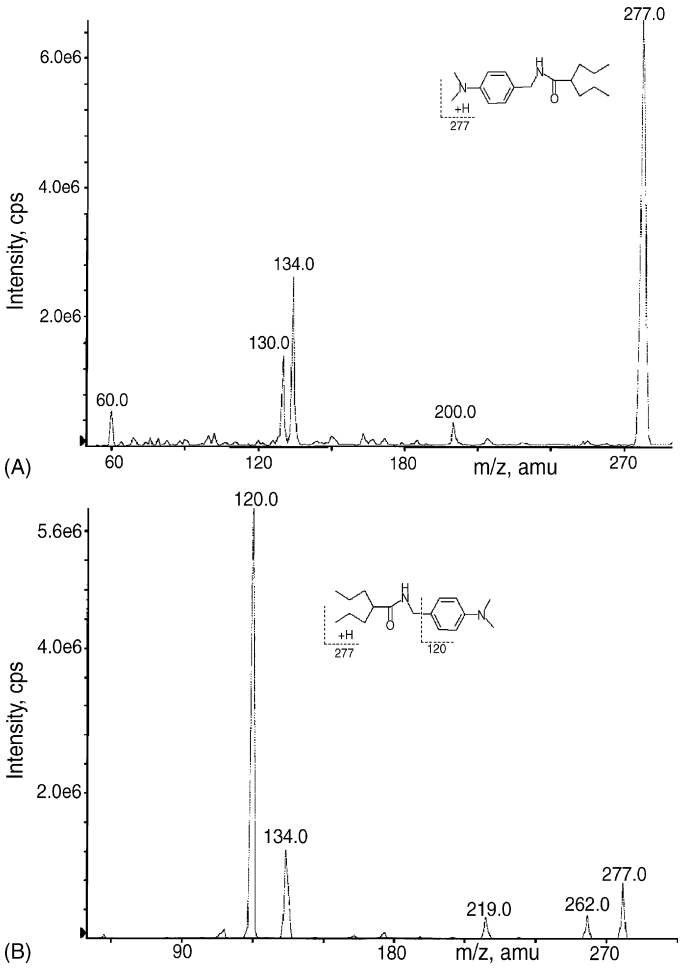
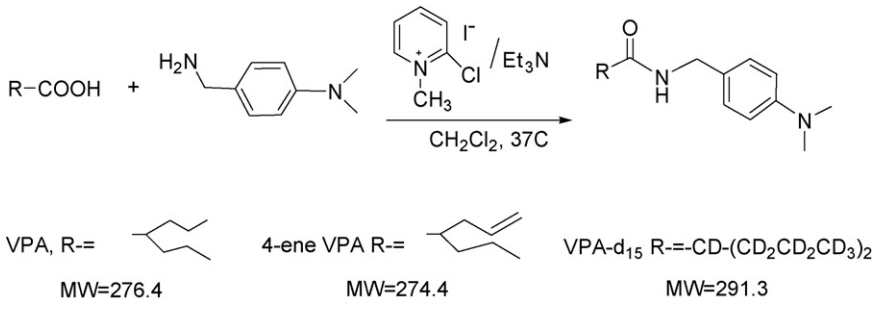


Fig. 2. Mass spectrum of the VPA derivative (A) and the product ion mass spectrum of ion at *m/z* 277 (B).

reported limit of determination of the HPLC-UV method was 2.5 μg/mL [13] and 1.25 μg/mL [14]. Further, direct measurement of these compounds with LC-MS following isolation is also not suitable due to the high background. In contrast, derivatization provided a possible way to quantitatively analyze this compound. Several methods have been previously developed based on chemical derivatization using GC-MS or HPLC method [9–11]. The derivatization reagents used in those studies include *para*-bromophenacyl bromide [11], *N*-trimethylsilyl-*N*-methyl-trifluoracetamide [15] pentafluorobenzyl bromide [16], and 2-(2-naphthoxy)ethyl-2-(piperidino)ethanesulfonate [17].



Scheme 1. Derivatization scheme of VPA, 4-ene VPA and VPA-d_{15}.

Other efforts, such as to reduce background interferences [16] and to optimize extraction procedure [9,15], have also been made to increase the sensitivity of quantification. However, the sensitivity, ranging from 0.5–10 μg/mL, was inadequate and appeared not to be adaptable to a LC-MS assay. Thus, there is no validated method available for quantitative determination of 4-ene VPA. Furthermore, the use of 4-dimethylamino-benzylamide derivatives to quantify VPA has not previously been reported. The derivatization was accelerated by activation of the carboxyl group using 1-chloro-4-methylpyridinium iodide and triethylamine (CMPI/TEA) [18]. The derivatization yielded a stable amide linkage (Scheme 1).

3.1. LC/MS/MS assay

First, the pure compound VPA and its metabolite 4-ene VPA were used to evaluate the derivatization procedure. The products were purified through a TLC plate (Analtech TLC Uniplates, Aldrich) and characterized by mass spectrometry (MS) and proton nuclear magnetic resonance (NMR). The electrospray mass spectrum of the VPA derivative is shown in Fig. 2A. The MH^+ ion of the derivatized VPA was detected at *m/z* 277. When the MH^+ was subject to collision activated dissociation (CAD) condition, it generated a base fragment ion at *m/z* 120 (Fig. 2B). The possible structures of these fragments are shown. The mass spectra of the derivatives of VPA-d_{15} and 4-ene VPA showed MH^+s as the base peak at *m/z* 292.0 and 275.0 (Figs. 3A and 4A), respectively. Their respective CAD mass spectra showed a base fragment ion at *m/z* 120 (Figs. 3B and 4B). The precursor/product ion pairs at *m/z* 277/120 and 275/120 were thus selected in the MRM mode for quantitation of the VPA and 4-ene VPA derivatives, respectively. The precursor/product ion pair at *m/z* 292/120 for the internal standard d_{15}-VPA derivative was also selected for the assay.

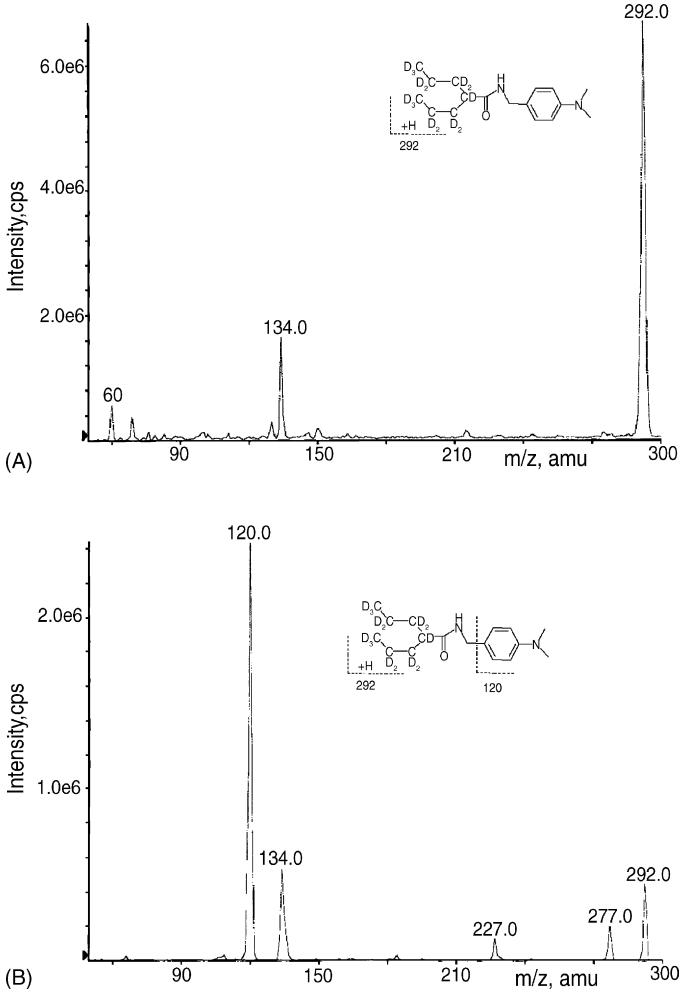


Fig. 3. The mass spectrum of the VPA-d_{15} derivative (A) and the product ion mass spectrum of ion at *m/z* 292 (B).

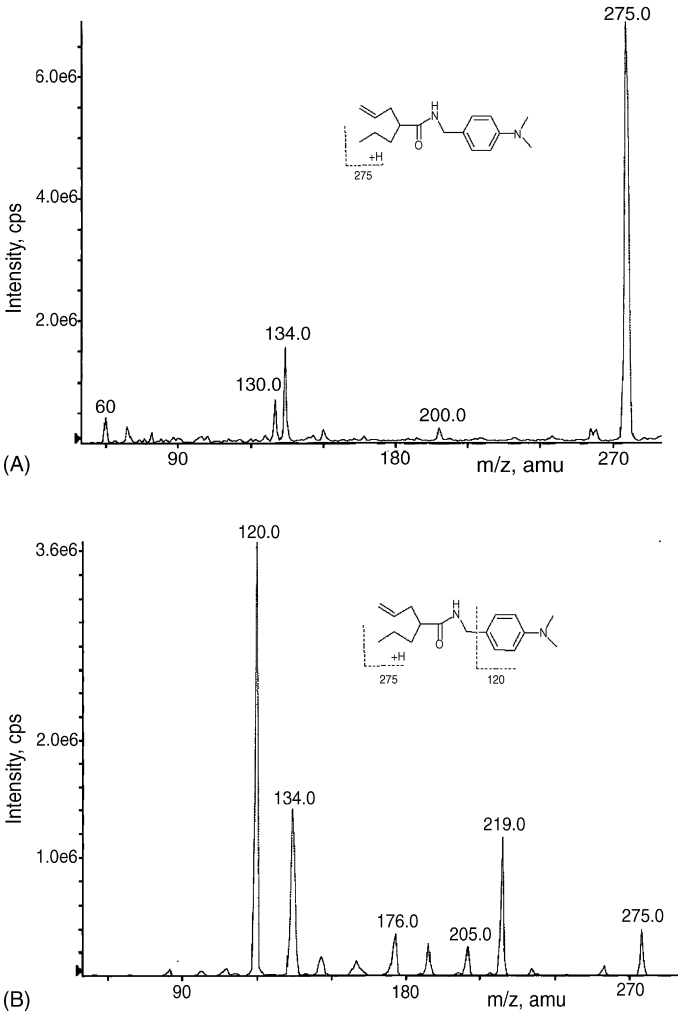


Fig. 4. The mass spectrum of 4-ene VPA derivative (A) and the product ion mass spectrum of ion at *m/z* 275 (B).

For the NMR data, the main features consistent with the literatures [19,20] that characterized similar structures of these derivatives include the resonances of: (i) the aromatic protons at δ 6.69–6.71 (d) and 7.13–7.16 (d); (ii) the amide hydrogen at δ 5.53–5.63 (s); (iii) the methylene attached to benzene at 4.33–4.36 (d); (iv) the protons of two methyl group attached to nitrogen atom at 2.94 (s); (v) the ethylene protons in 4-ene VPA derivative appearing at 4.99–5.08 (d,d) and 5.69–5.79 (m); (vi) the alkyl hydrogen atom of VPA derivatives appearing as multi-

ple peaks at high field (0.87–1.99), which are absent in VPA-d_{15} derivatives. The yield for VPA derivatization was found to be 45% based on 5 μg/mL concentration. Due to the low pK_a values (4.7–4.8) of VPA and 4-ene VPA free acid and high water solubility of their salt form, the biological samples needed to be acidified prior to extraction with an organic solvent. Acidification of the biological samples to pH 3 was necessary before solvent extraction. Following extraction, the derivatization must be performed in the extract solution directly, because evaporation of the extract solution would result in a significant loss of VPA and 4-ene VPA due to their volatile nature. We evaluated different solvents for extraction including acetonitrile, ethyl acetate, *tert*-butyl ether and dichloromethane and have found dichloromethane to be the most suitable, and the extraction recoveries were found to be 104 and 98% for VPA and 4-ene VPA, respectively. The derivatization reaction was performed in 1 h at 37 °C. The yield of derivatization reaction was found to be 45.6% based on a 10 μg/mL VPA concentration. Due to the structural similarity of these derivatives, their separation using an isocratic mobile phase could not be achieved. Therefore, a gradient elution system was used for the separation as described in the method section. Under the conditions we used, 4-ene VPA and VPA derivatives were well separated. As shown in Fig. 5, the retention times of VPA derivative, 4-ene VPA derivative and VPA-d_{15} derivative were found to be 7.09, 4.86 and 6.72, respectively. The peak area ratios between the analytes and the internal standard against the concentrations were used to generate the calibration curves. The peaks appearing before 4 min and after 10 min were identified as interference peaks.

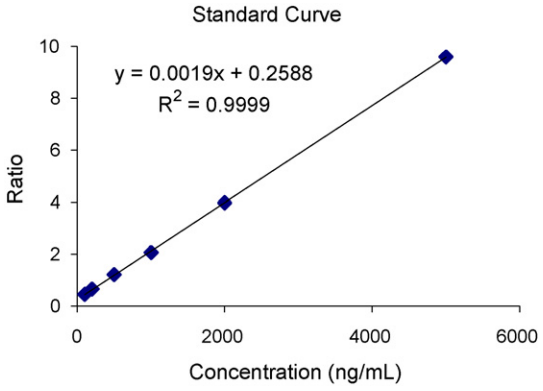


Fig. 6. Standard curve of VPA in human plasma.

3.2. Assay validation

The assay was validated in human plasma based on 0.1 mL plasma sample. The lower limits of quantification (LLOQ) for VPA and 4-ene VPA were found to be 200 and 20 ng/mL, respectively. Linearity was demonstrated between LLOQ to 5000 ng/mL for VPA (Fig. 6) and to 500 ng/mL for 4-ene VPA (Fig. 7). These sensitivity values are adequate for detecting clinically achievable levels. As shown in Tables 1 and 2, for VPA, the within-day coefficients of variation (CVs) were found to be 1.4,

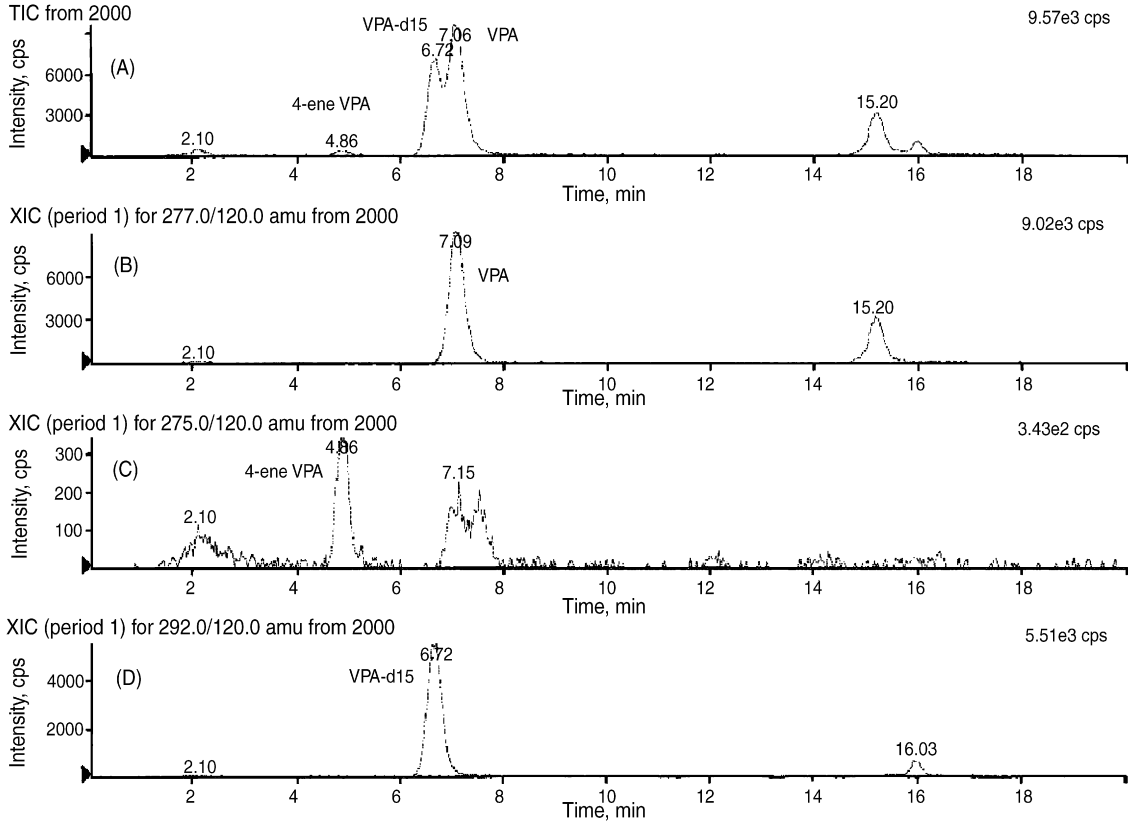


Fig. 5. The total ion chromatograms (TICs) of a processed plasma sample spiked with 200 ng/mL 4-ene VPA, 2000 ng/mL VPA and 1000 ng/mL VPA-d_{15} (I.S.) (A), the extracted ion chromatograms (XIC) of VPA (B), 4-ene VPA (C) and VPA-d_{15} (D).

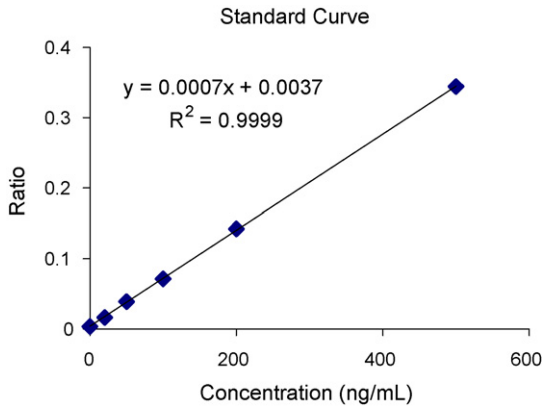


Fig. 7. Standard curve of 4-ene VPA in human plasma.

Table 1
Within-day and between-day validation characteristics of sodium VPA in 0.1 mL human plasma by API LC-MS/MS

	Concentration (mM (μg/mL))		
	1.2 (200)	6.0 (1000)	30.1 (5000)
Within-day			
Average ($n=6$)	1.2 (207.3)	5.9 (985.5)	30.6 (5078.9)
S.D.	0.02 (2.8)	0.4 (68.3)	0.4 (74.5)
CV (%)	1.4	6.9	1.5
Accuracy	103.6	98.6	101.6
Between-day			
Average ($n=6$)	1.2 (202.3)	6.1 (1003.5)	30.3 (5020.3)
S.D.	0.13 (21.4)	0.13 (32.8)	0.6 (99.0)
CV (%)	10.6	3.3	2.0
Accuracy	101.2	100.4	100.4

6.9, and 1.5% at 200, 1000, and 5000 ng/mL in human plasma ($n=6$), respectively. The between-day CVs for VPA were 10.6, 3.3, and 2.0%, respectively ($n=6$). For 4-ene VPA, the within-day CVs were 8.7, 2.9 and 2.3% ($n=6$) at 20, 100, and 200 ng/mL in human plasma, respectively (Table 2). The between-day CVs for 4-ene VPA were 6.7, 5.2 and 3.6%, respectively ($n=6$).

Table 3
Concentrations of 4-ene VPA in human plasma spiked with a high concentration of sodium VPA (100 μg/mL)

Nominal concentration (μM (ng/mL))	Observed concentration (μM (ng/mL))
0.14 (20)	0.14 (19.9)
0.35 (50)	0.35 (49.9)
0.70 (100)	0.72 (102.8)
1.41 (200)	1.41 (201.1)

3.3. Interference test

Quantification of 4-ene VPA was performed in the presence of 100 μg/mL sodium VPA. The data (Table 3) indicated that the presence of high concentration of VPA did not interfere with the quantification of 4-ene VPA. This could be accurately calculated from the standard curve as low as 20 ng/mL, therefore proving that simultaneous quantification of VPA and its hepatic metabolite 4-ene VPA is possible.

3.4. Application of the method to characterize the Preliminary Pharmacokinetics of VPA and 4-ene VPA in AML patients

This method was applied to quantify the plasma concentration in patients receiving VPA at 15–25 mg/kg in our clinical trial. The samples from AML patients dosed at 20 mg/kg were analyzed using 100 μL plasma. As shown in Fig. 8, following an oral dose at 20 mg/kg in a patient, plasma VPA concentrations increased continuously until 8 h (panel A), the last collected sample and there was substantial residual VPA concentrations before each of the subsequent dose (panel B). These results are consistent with the published data [21]. The metabolite, 4-ene VPA, was undetectable at the first dose until 8 h. After a daily dose of VPA for 5 days, the plasma concentration of VPA reached a steady state at about 80 μg/mL and 4-ene VPA concentrations were detected and also reached a steady state at 250 ng/mL in this patient. At steady state, 4-ene VPA was about 0.3% of that of VPA and its profile was essentially in parallel to that of VPA. Those data indicated that 4-ene VPA was accumulated in the body with the increasing VPA

Table 2
Within-day and between-day validation characteristics of 4-ene VPA in 0.1 mL human plasma by API LC-MS/MS

	Concentration (μM (ng/mL))			
	0.14 (20)	0.35 (50)	0.70 (100)	1.41 (200)
Within-day				
Average ($n=6$)	0.14 (20.3)	0.36 (51.5)	0.64 (91.4)	1.37 (194.5)
S.D	0.01 (1.8)	0.03 (3.6)	0.02 (2.6)	0.03 (4.6)
CV (%)	8.8	7.1	2.9	2.3
Accuracy	101.5	103.0	91.4	97.3
Between-day				
Average ($n=6$)	0.14 (20.1)	0.34 (48.6)	0.69 (97.6)	1.38 (196.5)
S.D	0.01 (1.4)	0.01 (1.4)	0.04 (5.0)	0.05 (7.0)
CV (%)	6.7	2.9	5.2	3.6
Accuracy	100.6	97.2	7.6	98.3

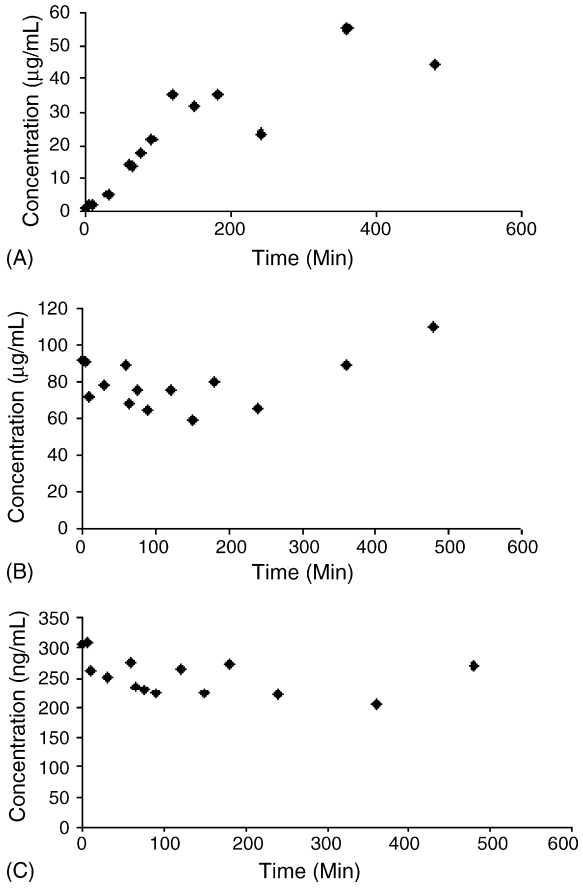


Fig. 8. Plasma concentration–time profiles of sodium VPA in one patient after given sodium VPA at 20 mg/kg/day: (A) after the first dose, (B) after daily dose for 5 days, and (C) plasma concentration–time profile of 4-ene VPA at day 5.

concentrations after an oral dose of sodium VPA and reached its steady state along with that of VPA.

4. Conclusion

A specific and sensitive method for the quantitative determination of VPA and 4-ene VPA in human plasma has been developed with good reproducibility and accuracy. The presence of high concentrations of VPA does not interfere with the quantification of 4-ene VPA. This makes the simultaneous quantification of VPA and its hepatic metabolite 4-ene VPA possible. This method has been applied to the analysis of VPA and its metabolite 4-ene VPA in clinical samples of acute myeloid leukemia (AML) patients treated with decitabine in combination with VPA.

Acknowledgements

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